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Translated by Sp/6 Charles T. Ostertag Jr.

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THE LAG PHASE AND OXIDATION-REDUCTION POTENTIAL IN CULTURES OF ANAEROBES

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The Lag Phase and Oxidation-Reduction Potential in Cultures of Anaerobes

I. L. Rabotnova and N. A. Pryanishnikova

As is known, the life cycle of bacteria may be divided into several stages or phases: 1) the lag phase - a period of growth inhibition which is always observed upon inoculation in a fresh medium; during this period the cells that have been introduced do not multiply, but in some manner prepare themselves for the subsequent intensive division; 2) logarithmic phase - a period of intensive multiplication; 3) stationary phase - the period of maturity of the culture; at this time the dying off of one portion of the cells has already begun but is compensated for by the multiplication of another; 4) the senile period - the progressive dying away of the culture.

The lag phase in bacteria has been investigated in numerous works, mainly by medical bacteriologists [2]. It has been clarified that it is dependent on many causes. The age of the inoculum is of great importance. The older the inoculant, the longer the lag phase. The greater the quantity of cells in the inoculant, the shorter the lag phase. The nature of the organism also has a great significance. Some bacteria begin to multiply very rapidly, for example *Bact. coli*, others such as the mycobacteria develop very slowly. There is an enormous importance in the conditions for cultivation: The composition of the medium, the pH, and the temperature. Under favorable conditions the lag phase is minimal, under unfavorable conditions it may be greatly extended.

The reason for growth inhibition immediately after inoculation is not clear. At any rate there are a number of suppositions explaining the presence of a lag phase. Some suggest that the bacteria have to somehow change the medium to a favorable condition by secreting some substance in it. Or the cells themselves have to change somehow, for example, store up the necessary reserve of some metabolites before they can proceed to division, or conversely, liberate the harmful exchange products accumulated during the preceding cultivation. There is also the proposal that before the beginning of division the cells have to create a specific level of carbon dioxide saturation of the medium or the cell itself.

However, all the enumerated proposals do not explain comprehensively the reasons for growth inhibition. It is clear only that during the lag phase the cells somehow become adapted to the new conditions and change the medium in accordance to their requirements.

Undoubtedly, among the conditions which should be provided for normal multiplication, the oxidation-reduction conditions have a great importance.

Experiments presented in a previous report [1] showed that cultures of anaerobes begin multiplication only when the cells lower the high oxidation-reduction potential. It can be assumed that the lag phase is a period necessary for the bacteria to establish a favorable rH_2 in the medium. If this is so, the addition of reducing agents in the medium should shorten the lag phase and the addition of oxidizing agents should lengthen it.

A series of experiments have been conducted for verifying this proposal.

Experiments with Putrefactive Anaerobes

Bac. sporogenes was cultivated in MPB + 0.2% of agar (MPB = meat peptone broth). During the period of development of the culture, the rH_2 was determined and the number of cells was calculated by the direct count method. Along with this, observations were carried out on the morphology of the culture. Comparisons were made between cultures with an addition of a reduction agent - hydrosulfite, and those without a reduction agent.

As is known, the duration of the lag phase depends on the age of the inoculum. Therefore in the first series of experiments we conducted the inoculations from cultures of Bac. sporogenes of various ages. Each hour samples were taken for a computation of cells and the rH_2 was measured. After sterilization, 0.013% hydrosulfite was added. The results are depicted in figure 1. As is apparent from the charts, the older the inoculum, the longer the lag phase. In all cases multiplication begins only when the rH_2 is lowered to 2-3 by the culture itself, that is, extremely low. The addition of hydrosulfite when inoculating with a 6-hour culture almost completely liquidates the lag phase, and when inoculating with an older culture considerably shortens it. The microscopic nature of a culture on a medium with hydrosulfite is depicted with the help of a drawing device in figure 2.

Analogous experiments were carried out with various amounts of inoculum. The inoculation was carried out with a 14-hour culture in the amounts of 0.1, 0.3, 0.5, and 0.7% which constituted the corresponding number of cells: 3.3, 4.1, 7.6, and 10 million per ml. In parallel test tubes 0.013% of hydrosulfite was added. The results are depicted in figure 3.

As can be seen, with a small amount of inoculum - 0.1% - the lag phase is very much extended: After 6 hours multiplication still hasn't begun. With an inoculum of 0.7% the lag phase is 3-4 hours. The addition of hydrosulfite in this case completely eliminates the lag phase, and with a small amount of inoculum considerably shortens it.

Even though the lag phase is cut when the rH_2 is lowered with hydrosulfite, the stage of long filaments remains. In the experiment with hydrosulfite, filaments are apparent after 1 hour; after two and one half hours they have already broken up into chains and separate cells.

With the addition of thionine to the medium, it is buffered at a high level ($rH_2 = 16$) and the lag phase is extended to many hours.

Thus, it can be concluded that the duration of the lag phase is determined to a considerable degree by the oxidation-reduction properties of the medium.

Ascorbic acid and in part glucose act similar to hydrosulfite - they shorten the lag phase.

Experiments with Butanol-Acetone Forming Bacteria

Analogous investigations of the lag phase were conducted with butanol-acetone forming bacteria. The microorganisms were incubated in a 15% potato mash with 2% agar. The addition of agar is necessary because during a frequent sampling of specimens, oxygen diffuses easily into the unthickened medium and disrupts the normal development of the culture. The inoculation was done with two percentages of a culture (10-12 million cells in 1 ml) under conditions of active fermentation. Experiments, based on the specific rH_2 and multiplication of cells, showed that the lowering of the rH_2 takes place very rapidly during the course of development of the culture. After 1-3 hours, the rH_2 is lowered to almost 0. Cell multiplication begins with somewhat of a greater delay than in Bac. sporogenes. The logarithmic phase of growth begins after 4-5 hours. Apparently these bacteria are more anaerobic than putrefactive bacteria, and to a greater degree they require a saturation of the medium with reducing substances. Nevertheless butanol-acetone forming bacteria can overcome a high oxidation-reduction potential. In one experiment, with the introduction of 0.4% of inoculum, the initial rH_2 was 21.3 and after six and one half hours it dropped to 0.4. Shortly after this cell multiplication began.

The addition of 0.01 - 0.013% of hydrosulfite exerted a speeding up action on the development of the culture (fig. 4).

Observations on the morphological properties of the cells showed that during the lag phase they increased in size. However this is expressed less sharply than in putrefactive bacteria. Normal cells are encountered along with elongated cells and a strong elongation is not observed.

Experiments with Ethyl Alcohol-Acetone Forming Bacteria

Ethyl alcohol-acetone forming bacteria were cultivated in a 15% potato mash without thickening the medium, in as much as they, being facultative anaerobes, do not suffer on account of oxygen infiltration into the medium.

Observations of the rH_2 and multiplication showed that multiplication of cells begins with a comparatively small lowering of the potential. At a $rH_2 = 12-19$ an increase in the number of cells is already taking place (5-7 hours after inoculation), and the rH_2 continues to drop and after 7-8 hours reaches the low values of 1-2.

The ethyl alcohol-acetone forming bacteria do not have a stage of cell elongation. Only a small increase in size takes place. Some polymorphism is characteristic for these bacteria. The length of the cells in all ages is various.

The addition of 0.01% of hydrosulfite lowers the rH_2 in the medium and speeds up cell multiplication just as in experiments with strict anaerobes (figs. 6 and 7).

Conclusions

Having established earlier that anaerobic microorganisms can multiply only under extreme reduction conditions created by themselves, we proposed that, to a considerable degree, the reason for the growth inhibition phase is an inadequate oxidation-reduction potential. In reality, by adding hydrosulfite in a quantity that still isn't toxic but lowers the initial potential of the medium, the lag phase in Bac. sporogenes can be shortened. This is visually apparent when inoculation is carried out from an old culture or with a small amount of cells. However the stage of gigantism isn't completely left out by the addition of reduction agents. This testifies to the fact that the rH_2 isn't the only factor which causes growth inhibition. It goes without saying that growth inhibition depends on many factors and oxidation-reduction conditions are one of the most essential. The addition of oxidizers inhibits the lag phase.

Butanol-acetone forming bacteria are more strict anaerobes than putrefactive bacteria. They rapidly lower the rH_2 in a medium from 20-21 to almost 0, but multiplication begins with a greater delay than in putrefactive bacteria. The addition of hydrosulfite shortens the duration of the lag phase in butanol-acetone forming bacteria.

Facultative anaerobic ethyl alcohol-acetone forming bacteria begin to multiply at the comparatively high values of $rH_2 = 12-19$. During the multiplication of cells the rH_2 drops down to 1-2. There is no gigantism stage in them. The addition of hydrosulfite speeds up multiplication even in ethyl alcohol-acetone forming bacteria. This testifies to the fact that though they are facultative anaerobic organisms, the optimum conditions for them are a somewhat reduced state in the medium.

Results

The lag phase in the development of cultures of strict anaerobes - Bac. sporogenes and Cl. acetobutylicum and the facultative aerobe Bac. acetoethylicus - can be shortened by adding reducing agents - hydrosulfite or ascorbic acid - and lengthened by adding oxidizers.

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Literature

1. Rabotnova, I. L., Toropova, Ye. G., Rabayeva, M., Requirements of

Anaerobic Bacteria in Oxidation-Reduction Conditions of a Medium, Microbiology, XXIV, #5, 1955.

2. Porter, J. R., Bacterial Chemistry and Physiology, N. Y., 1946.

Figures

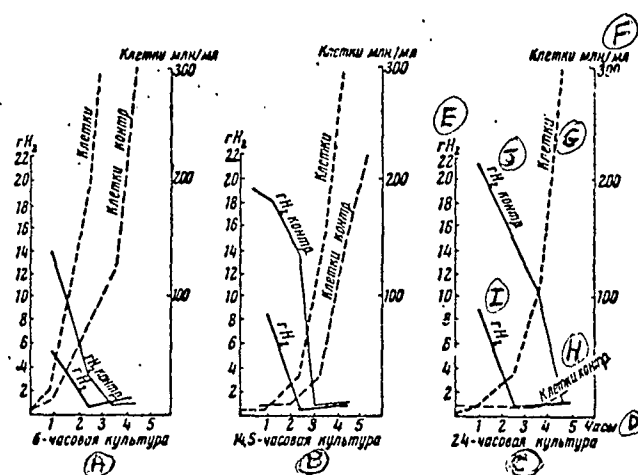


Рис. 1. Влияние гидросульфита на лаг-фазу *Bac. sporogenes* при засеивании посевным материалом различного возраста

Figure 1. The influence of hydrosulfite on the lag phase of *Bac. sporogenes* during seeding with inoculum of various ages.

- | | |
|----------------------|----------------------------|
| A. 6-hour culture | F. cells, million/ml |
| B. 14.5-hour culture | G. cells |
| C. 24-hour culture | H. control cells |
| D. hours | I. rH ₂ |
| E. rH ₂ | J. control rH ₂ |

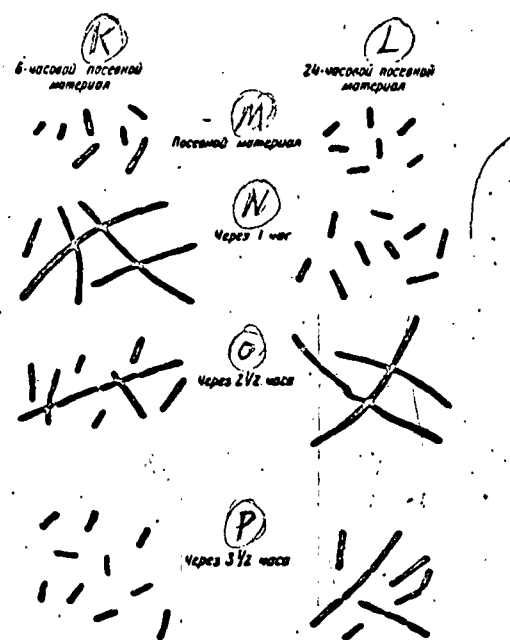


Figure 2. Bac. sporogenes in a medium of $\text{Na}_2\text{S}_2\text{O}_4$.

- K. 6-hour inoculum
- L. 24-hour inoculum
- M. inoculum
- N. after 1 hour
- O. after 2.5 hours
- P. after 3.5 hours

Рис. 2. Bac. sporogenes на среде с $\text{Na}_2\text{S}_2\text{O}_4$.

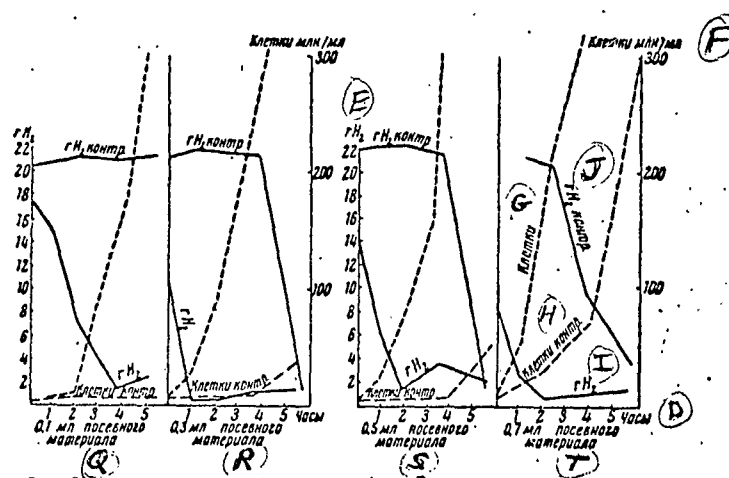


Рис. 3. Влияние гидросульфита на лаг-фазу Bac. sporogenes при внесении различного количества посевного материала

Figure 3. Influence of hydrosulfite on the lag phase of Bac. sporogenes with the admission of various quantities of inoculum.

- Q. 0.1 ml of inoculum
- R. 0.3 ml of inoculum
- S. 0.5 ml of inoculum
- T. 0.7 ml of inoculum

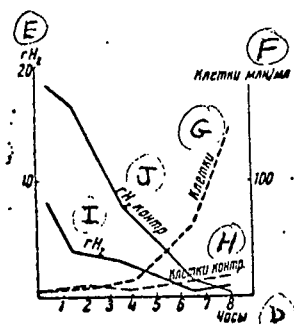


Рис. 4. Влияние гидросульфита на лаг-фазу у ацетобутиловых бактерий. 1% посевной материал. 0,01% гидросульфита

Figure 4. Influence of hydrosulfite on the lag phase of butanol-acetone forming bacteria. 1% of inoculum. 0.01% of hydrosulfite.

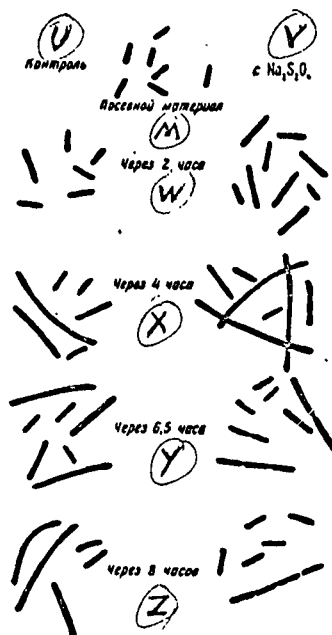


Рис. 5. Ацетобутиловые бактерии

Figure 5. Butanol-acetone forming bacteria.

- U. control
- V. with $\text{Na}_2\text{S}_2\text{O}_4$
- M. inoculum
- W. after 2 hours
- X. after 4 hours
- Y. after 6.5 hours
- Z. after 8 hours

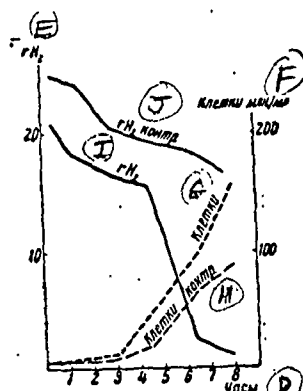


Рис. 6. Влияние гидросульфита на лаг-фазу у ацетоноэтиловых бактерий

Figure 6. Influence of hydrosulfite on the lag phase of ethyl alcohol-acetone forming bacteria.



Рис. 7. Ацетоноэтиловые бактерии

Figure 7. Ethyl alcohol-acetone forming bacteria.

M. inoculum
W. after 2 hours
X. after 4 hours
Za. after 5 hours